Remarks

Applicants request entry of the amendments and reexamination. The amendments place the application in condition for allowance.

The first line of the claims section of the specification has been amended to recite the words "We claim:" as suggested by the Examiner.

Claims 2 and 21 are amended to deleted one of the appearances of the word "or".

Claims 3 and 22 are amended to recite the CTLA4Ig molecule in a manner that distinguishes it from the recited antibodies.

No new matter enters by these amendments.

The Examiner states that a certified copy and translation of the French priority document has not been filed. However, in the parent application, the certified translation was filed on January 7, 2000. An additional copy for the Examiner's convenience is enclosed. Furthermore, the Notification of Acceptance for the U.S. National Stage parent application indicates that the priority document was received by the U.S. Patent Office (copy of Notification enclosed). Accordingly, having previously met all the requirements for a proper claim to priority, applicants respectfully request benefit of all the claimed priority applications.

The Examiner notes errors in the previously filed forms accompanying the Information Disclosure Statement. As it seems all of the documents marked as "not considered" have been included on the Examiner's "Notice of References Cited" pages accompanying the Office Action, applicants conclude that the Examiner has already considered all of the documents submitted. Clarification is requested if this understanding is not correct. Each of the three foreign documents have been signed as "considered" on 3-5-07. Accordingly, applicants conclude that the foreign documents have also been considered. If this is incorrect, applicants respectfully request clarification.

Claims rejected under 35 U.S.C. § 112, second paragraph

The Examiner considers the language in claims 2 and 21 vague. Applicants assert that one of skill in the art reading the claim would understand that the immunosuppressive

agent could be selected from the list including the recited chemical compounds as well as the recited antibodies. Applicants have removed the first "or" in claim 2 and 21 to attempt to address the Examiner's confusion. However, applicants assert that one of skill in the art would have no difficulty reading and understanding claim 2 or 21 in either the original or amended version.

The Examiner indicates it is unclear if the molecule CTLA4Ig is included in the antibodies of claim 3 and 22. Applicants have amended claims 3 and 22 to clarify the immunosuppressive agent, the antibodies, and have made claim 3 dependent upon claim 1.

The Examiner states that it is unclear what the terms CD4, ICAM-1, LFA-1, B7, and CTLA4Ig refer to in claims 3 and 22. The specification on several pages refers to "anti-CD4, -CD2, -CD8, -CD28, -B7, -ICAM-1 and -LFA-1 antibodies" (see page 7 at lines 26). As noted in the specification, these antibodies are directed against lymphocyte antigens. Applicants submit that one of skill in the art was familiar with examples of these antibodies. As evidence, applicants submit an excerpt of pages from the text "Fundamental Immunology," Third Edition, published in 1993, which refers specifically to many of these antibodies. Similarly, applicants submit that one of skill in the art was familiar with the abbreviations aFGF and bFGF for acidic and basic Fibroblast Growth Factor. Also, the specification refers to CTLA4Ig at page 10, lines 1-7. Applicants request reconsideration. While the complete spelling of the words for these abbreviations can be inserted into the claims, applicants submit that it is not necessary for one of skill in the art to understand what is claimed. The fact that these abbreviations are known in the art suffices under the circumstances.

Applicants request that these rejections under 35 U.S.C. § 112, second paragraph, be withdrawn.

Claims rejected under 35 U.S.C. § 112, first paragraph

Claim 1-5, 7-26, 28-34, and 36 stand rejected under 35 U.S.C. § 112, first paragraph, as the specification allegedly fails to enable the scope of the claims. Applicants respectfully disagree.

Claims 1-5, 7-18, and 34 each recite a composition. It is not clear that any of the reasoning in the rejection under this section acknowledges that the compositions need not be used to cure a disease by a gene therapy method. A composition, and any other claimed invention, need not comply with the therapeutic efficacy and safety requirements of the FDA in order to be patentable. The compositions can be used in research and development. One of skill in the art would know, for example, that anti-CD4 antibodies were the subject of past clinical trials and considerable research and development. Combining those antibodies with the recited recombinant adenovirus would similarly be useful in research and development and one of skill in the art would certainly know how to combine these two agents.

The Examiner states at one point that "the claims read on gene therapy in vivo in light of the specification." However, claims 1-5, 7-18, and 34 recite a composition. One use could be in vivo gene therapy. However, the compositions can also be used for in vitro or other methods or studies. Therefore, there would appear to be no reason to require applicants to show a "therapeutic effect" as the Examiner does in the Office Action.

Furthermore, the ability to make and use the compositions claimed would not require trial and error experimentation. Given the direction supplied by the specification and the availability of the immunosuppressant agents to one of skill in the art, producing the compositions would be simple. Similarly, using the compositions for research and development, even for in vitro studies, would require no trial and error.

With respect the method claims including claims 19-26 and 28-33, applicants respectfully submit that the evidence in the specification suffices for one of skill in the art to make and use the claimed invention. Applicants point out that the gp19k sequence recited in the claims could have been selected from a number of sequences known in the art. The parent application refers to two examples from the published papers available to one of skill in the art at the time this application was filed (Flomenberg et al., J. of Virol. 62:4431-4437 (1988) and Hermiston et al. Virology 197:593-600 (1993)). Both of these papers show the sequence of a gp19k protein from adenovirus that could have been selected without undue experimentation. Applicants have also shown in the specification at, for example, page 13, line 9 through page 14, line 5, and Example 2.3 beginning at page 42, that the

advantageous characteristics of the recited adenoviral vectors do work as asserted. Mice administered an adenoviral vector of the claimed invention show a lack of inflammatory reaction and a prolonged expression period (see page 43, lines 6-14). This aspect of the invention is recited in claim 38, for example. As one of ordinary skill in the art would appreciate, this property improves the longevity and/or persistence of adenoviral vector in infected cells. Applicants demonstrated the improved persistence in vivo in an acceptable model system. No evidence shows or suggests that this demonstration is incorrect or that the general properties of the vectors shown in this demonstration cannot be used by others.

The Examiner also cites articles that refer generally to gene therapy clinical efficacy, like Verma and Eck and others, to support the argument that gene therapy is an unpredictable art and the conclusion that trial and error experimentation are required to make and use the claimed invention. These articles address the clinical effectiveness, optimization of efficacy, and other clinical considerations for gene therapy (see page 239, first paragraph of Verma, and Table 5-1 of Eck). The citation to articles that relate to clinical standards -- which are the realm of the FDA, and not the PTO standard -- at least implies that the only evidence sufficient to dispel the concerns of these articles is clinical trial evidence. Clearly, that is the type of evidence treated in these papers. Regardless, nothing in these papers says, or would be taken by one of skill in the art to say, that gene therapy is devoid of promising inventions or devoid of any patentable, pharmaceutical properties akin to those discussed in *In re Brana*, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). The *In re Brana* decision reversed a PTO rejection that improperly applied an FDA-like standard to an anti-tumor compound instead of the appropriate patent law standard.

Viewing all of applicants' arguments and evidence demonstrating enablement, applicants submit that the claims meet the statutory standard for enablement under 35 U.S.C. § 112, first paragraph. Furthermore, the specification contains a detailed description of how to make and how to use a composition comprising an immunosuppressive agent and a recombinant adenovirus whose genome comprises a first recombinant DNA comprising a sequence of interest and a second recombinant DNA containing a sequence coding for adenoviral gp19k protein. Therefore, applicants submit

that this rejection is untenable and respectfully request that it be reconsidered and withdrawn.

In addition, applicants request an opportunity to discuss the application with the Examiner in an interview and applicants' representative will contact the Examiner.

Rejections under 35 U.S.C. §103

Applicants note at the outset the request and showing above related to the proper priority date for the instant application. The Wilson document (5,872,154) at least appears to be unavailable as prior art against the claims in light of this priority claim.

Claims 1-5, 7-17, 19-23, 25, 26, 28-34 and 36 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Leibowitz in view of Pearson and Nabel. Applicants respectfully disagree.

The Examiner cites Leibowitz for an adenovirus containing gp19K and Pearson for anti-CD4 antibody. Neither suggests the compositions claimed and recited.

Apparently, the Nabel document is cited to bring the prior two documents together and form a motivation to combine the teachings. However, the statements in Nabel are first opposite to those cited from Verma and Eck above (see pages 248-9 of Nabel) concerning the prospects and use of adenoviral vectors for gene therapy, and then Nabel states that issues related to "stability of expression" must be explored (page 250). There is nothing in Nabel referring to gp19k as a direction for exploration in particular. Furthermore, there is nothing in any of these documents suggesting the prolonged expression resulting from the combination compositions recited and claimed herein. The motivation to find a successful result, for example with adenoviral vectors, will always be present. But in this case, the motivation to specifically use gp19K sequences in combination with an immunosuppressive agent cannot be extracted from any reading of these documents. In addition, the conclusion at page 15 of the Office Action referring to MHC class I seems to have nothing to do with the recited combination but only to do with Leibowitz alone.

There is certainly no "clear and particular" showing for the motivation to combine anywhere from these documents or the Examiner's comments about the documents. *See Ruiz v. A. B. Chance Co.* 57 U.S.P.Q.2d 1161 (Fed. Cir. 2000). The Patent Office should

lay out on the record the required specificity for a motivation to combine assertion. *In re Lee*, 61 U.S.P.Q.2d 1430 (Fed. Cir. 2002). Alternatively, some reason to arrive at the improved results shown in the specific manner claimed should be analyzed. Nothing from the cited documents provides a clear, specific, or particular motivation to combine in this case. Applicants respectfully submit that it is only illegal hindsight that allows a combination as presented in the rejection. Note that the requirement to show a specific motivation to combine is the safeguard against illegal hindsight analysis. *In re Lee*, 61 U.S.P.Q.2d 1430 (Fed. Cir. 2002), citing *In re Dance*, 48 U.S.P.Q.2D (BNA) 1635, 1637 (Fed. Cir. 1998).

Claims 1-5, 7-17, 19-23, 25, 26 and 28-33 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Leibowitz in view of Wilson. Applicants respectfully disagree.

Wilson was filed after applicants' priority date and was published over one year after applicants' priority date. Furthermore, applicant's French priority document refers to the recited gp19k protein sequence and the compositions claimed and recited herein.

Applicants submit that this rejection is improper and request its withdrawal.

Accordingly, applicants respectfully request that these rejections be withdrawn.

Conclusion

Applicants believe that this application is now in condition for allowance. If the Examiner believes that prosecution might be furthered by discussing the application with applicants' representative, in person or by telephone, we would welcome the opportunity to do so.

If there are any additional fees due with the filing of this document, including fees for the net addition of claims, applicants respectfully request that any and all fees be charged to Deposit Account No. 50-1129. If any extension of time request or any petition is required for the entry of this paper or any of the accompanying papers, applicants hereby petition or request the extension necessary. The undersigned authorizes any fee payment from Deposit Account No. 50-1129.

Respectfully submitted,

Wiley Rein LLP

Date: September 6, 2007

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NOTIFICATION OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

1. The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as ☐ a Designated Office (37 CFR 1.494), ☒ an Elected Office (37 CFR 1.495), has determined that the above identified international application has met the requirements of 35 U.S.C. 371, and is **ACCEPTED** for national patentability examination in the United States Patent and Trademark Office.

2. The United States Application Number assigned to the application is shown above and the relevant dates are:

22 MAY 1998
35 U.S.C. 102(e) DATE

22 MAY 1998
DATE OF RECEIPT OF 35 U.S.C. 371 REQUIREMENTS

A Filing Receipt (PTO-103X) will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371(C) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN ABOVE.** *The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363).* Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

3. ☒ A request for immediate examination under 35 U.S.C. 371(f) was received on 14 Aug 1997 and the application will be examined in turn.

4. The following items have been received:
- ☒ U.S. Basic National Fee.
- ☒ Copy of the international application in:
  - ☒ a non-English language.
  - ☐ English.
- ☐ Translation of the international application into English.
- ☒ Oath or Declaration of inventors(s) for DO/EO/US.
- ☐ Copy of Article 19 amendments. ☐ Translation of Article 19 amendments into English.
  The Article 19 amendments ☐ have ☐ have not been entered.
- ☐ The International Preliminary Examination Report in English and its Annexes, if any.
- ☐ Copy of the Annexes to the International Preliminary Examination Report (IPER).
  - ☐ Translation of Annexes to the IPER into English.
  The Annexes ☐ have ☐ have not been entered.
- ☒ Preliminary amendment(s) filed 14 Aug 1997 and _____.
- ☒ Information Disclosure Statement(s) filed 12 SEP 1997 and _____.
- ☐ Assignment document.
- ☐ Power of Attorney and/or Change of Address.
- ☐ Substitute specification filed _____.
- ☐ Statement Claiming Small Entity Status.
- ☒ Priority Document.
- ☒ Copy of the International Search Report ☒ and copies of the references cited therein.
- ☐ Other:

Applicant is reminded that any communication to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above. (37 CFR 1.5)

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FUNDAMENTAL IMMUNOLOGY

THIRD EDITION

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TABLE 3. *Anti-LFA-1 and anti-ICAM-1 mAbs block the adhesion of T lymphoblasts[a] to ICAM-1 incorporated into planar membranes*

Continuous presence of mAb	
mAb added[b]	Percentage of cells bound
None	28
Anti-LFA-1	2
Anti-ICAM-1	2
Anti-MHC class I	27
Anti-LFA-3	28

Pretreatment with mAb		
Pretreat[c]	mAb	Percentage of cells bound
T cells	—	40
T cells	Anti-LFA-1	2
T cells	Anti-ICAM-1	39
T cells	Anti-LFA-3	39
ICAM-1 membrane	—	39
ICAM-1 membrane	Anti-LFA-1	39
ICAM-1 membrane	Anti-ICAM-1	2
ICAM-1 membrane	Anti-LFA-3	38

Adapted from ref. 30.

[a] Radiolabeled T lymphoblasts were added to ICAM-1 incorporated into lipid vesicles and bound to plastic microtiter wells.

[b] The indicated mAbs were present throughout the experiment.

[c] Cells or the plastic-bound vesicles were pretreated with the indicated mAb and then washed to remove unbound antibody.

characteristics normally seen in LFA-1-dependent cell–cell interactions. Thus the binding of cells to ICAM-1-containing membranes required metabolic energy, a functional cytoskeleton, and the presence of Mg^{2+}. Single amino acid mutation studies have shown that the primary binding sites of contacts between ICAM-1 and LFA-1 are discontinuous amino acids in the first Ig domain. Human LFA-1 will interact with murine ICAM-1, but murine LFA-1 will not interact with human ICAM-1. This restriction in cross-species binding is due to the LFA-1 α chain, since mouse–human hybrids expressing human α chains and murine β chains will bind human ICAM-1.

ICAM-2

During studies of the binding of human lymphocytes, it was noted that an anti-ICAM-1 mAb could not inhibit all the LFA-1-mediated adhesion. This implied that a second ligand for LFA-1 existed on endothelial cells. ICAM-2 was found to be a broad band on SDS–PAGE of M_r 55,000 to 65,000 under reducing conditions (31). Unlike ICAM-1, little variation in size was seen for ICAM-2 immunoprecipitated from different cell lines. On cDNA sequencing, ICAM-2 has a polypeptide backbone of 28,393 and six potential N-linked glycosylation sites. ICAM-2 has only two Ig-like domains that are most homologous to the two N-terminal domains of ICAM-1 with 35% amino acid identity. The overall tissue distribution of ICAM-2 is more restricted than that of ICAM-1. ICAM-2 is restricted largely to endothelium and certain interstitial cells. ICAM-2 is not expressed on thymocytes. The level of ICAM-2 on resting endothelial cells is 10- to 15-fold higher than that of ICAM-1. ICAM-2 expression on resting lymphocytes was several fold higher than that seen with ICAM-1, while monocytes expressed equivalent levels of ICAM-1 and ICAM-2. While endothelial cell ICAM-1 expression is inducible by exposure to cytokines, ICAM-2 expression on endothelial cells is not affected by a variety of inflammatory cytokines. These results suggest that ICAM-1 is the major ligand for LFA-1 during inflammatory or immune responses while ICAM-2 is of more relative importance in the unstimulated resting state or early during a response before ICAM-1 expression is increased. ICAM-2 is also an attractive candidate to facilitate memory T cell recirculation as it is basally expressed at high levels on resting endothelium and memory T cells have increased LFA-1 expression. As resting T cells express very little ICAM-1, ICAM-2 may be important in the initial T cell adhesion with antigen-presenting cells that bear LFA-1. ICAM-2 has been shown to have a lower affinity for LFA-1 than ICAM-1. ICAM-1 also can interact with Mac-1, but ICAM-2 does not bind to Mac-1.

ICAM-3

Blocking studies with anti-ICAM-1 and anti-ICAM-2 mAbs suggested that a third ligand for LFA-1 might exist. Thus the homotypic adhesion of a T lymphoma line to purified LFA-1 could be blocked by anti-LFA-1, but not by anti-ICAM-1 or -2 (Table 4). A mAb has been generated to ICAM-3 (32), which differs in its tissue dis-

TABLE 4. *Effect of anti-ICAMs on adhesion of SKW3 cells to LFA-1[a]*

mAbs	Percentage of cells bound
Control	65
Anti-ICAM-1	64
Anti-ICAM-2	60
Anti-ICAM-3	38
Anti-ICAM-1 + 2	59
Anti-ICAM-1 + 3	28
Anti-ICAM-2 + 3	5
Anti-ICAM-1 + 2 + 3	4

Adapted from ref. 32.

[a] SKW3 cells were pretreated with the indicated mAb(s) and then allowed to bind to solid-phase LFA-1 for 1 hr at 37°C. Adhesion was not significantly inhibited by anti-ICAM-1 or anti-ICAM-2 separately or in combination. Anti-ICAM-3 alone significantly inhibited adhesion and complete inhibition was seen with the combination of anti-ICAM-2 and -3. Thus adhesion of SKW3 cells to LFA-1 is mediated mainly by ICAM-3 and to a lesser extent by ICAM-2.

TABLE 19. *CD44H raises while CD44E lowers tumor metastatic potential*[a]

Tumor injected	Percentage of animals with tumors
Namalwa CD44$^-$	40
Namalwa CD44H	80
Namalwa CD44E	20

Adapted from ref. 148.

[a] Animals were injected intravenously with CD44$^-$ Namalwa cells or with Namalwa cells transfected with the CD44H or CD44E antigens. Tumor growth was assessed histologically 80 days after inoculation.

activated. It thus appears that following T cell activation, stimulation of CD44 by mAb or by ligand may lead to further T cell activation and adhesion. This cascade of events may again be important in inflammatory sites where CD44 on partially activated T cells may interact with extracellular matrix constituents containing hyaluronic acid and lead to an augmentation of the inflammatory process.

The interaction of certain of the CD44 isoforms with hyaluronate appears to play a major role in determining the metastatic potential of tumor cells. Increased production of hyaluronate is associated with tumor growth and has been proposed to enhance tumor invasiveness. When the hematopoietic (CD44H) and epithelial cell isoforms (CD44E) of CD44 were transfected into a human Burkitt lymphoma cell line, which does not constitutively express CD44, only transfectants expressing CD44H adhered to hyaluronate-coated surfaces (148). When the transfectants were injected into nude mice, the expression of CD44H enhanced both primary and secondary tumor growth (Table 19). Animals injected with tumor cells expressing CD44E rarely developed tumors. It is likely that the expression of CD44H enhances secondary tumor growth by facilitating interactions between tumor cells and host tissues since each of the tissues in which development of CD44H tumors occurred had an elevated content of hyaluronate. Since CD44H and CD44E only differ in their extracellular domains and have identical cytoplasmic domains, the observed differences in their tumor-promoting properties are most likely due to the presence of the additional 134 residues in the extracellular domain of CD44E. Further evidence for a role of CD44H in tumor development was obtained from studies in which the growth of the CD44H transfectants could be suppressed by a soluble human CD44H Ig fusion protein (149).

CD28–CTLA-4 AND THEIR LIGAND B7

Tissue Distribution and Structure

The human CD28 antigen is identified with mouse mAb 9.3, which was one of the first mAbs to be generated against human peripheral blood T cells (150). The CD28 antigen is expressed on a large percentage of human peripheral T cells including 95% of the CD4 subset and 50% of the CD8 subset. The level of expression of CD28 can be enhanced by T cell activation (151). The CD28 antigen is not expressed on the subset of T cells that express the TCR $\gamma\delta$ chains (152). It has also been reported that those T cells that are CD28$^-$ are the only T cells in peripheral blood that express the CD11b antigen and react with the OKM1 mAb (153); the significance of this finding is obscure at present. Although it was originally stated that CD28 expression was T cell specific, it has been shown that plasma cells and anti-Ig-activated B cells can express CD28 (154).

The 9.3 mAb recognizes a disulfide-linked molecule, which consists of heavily glycosylated 44-kD subunits on SDS–PAGE. No evidence for a biochemical association between CD28 and the CD3/TCR complex has been shown. A cDNA for the CD28 antigen has been cloned (155) and it predicts a protein of 202 amino acid residues with a core peptide with a molecular weight of 23 kD, five potential N-linked glycosylation sites, and a 41 residue cytoplasmic domain. Human CD28 is encoded by a single copy gene organized into four exons and each exon defines a functional domain of the predicted protein. The amino acid sequence of CD28 displays substantial homology with mouse and rabbit Ig heavy chain variable regions over a large portion of the extracellular domain. Furthermore, two cysteine residues in this stretch are separated by approximately the same number of amino acids that separate the cysteine residues in related regions of CD4, CD8, and Ig heavy and light chain variable domains. These data establish CD28 as a member of the Ig supergene family. Following transfection of the CD28 cDNA into COS cells, mAb 9.3 precipitates a homodimer demonstrating that a single gene can encode the CD28 dimer. However, heterodimer formation for the native CD28 antigen complex has not been excluded.

A portion of the human CD28 cDNA has been used to isolate a homologous murine clone (156). The murine CD28 sequence shares a 61% nucleotide identity with the human cDNA and many characteristics of the human cDNA molecule are conserved in murine CD28. The murine CD28 sequence encodes a peptide of 218 amino acids; all five potential N-linked glycosylation sites are conserved and six of the seven cysteine residues of the mouse protein are found in the human CD28 polypeptide. Deglycosylation studies indicate that four of the five N-linked glycosylation sites are used and the mature core protein has a molecular mass of 25 kD, which is close to that predicted by the cDNA sequence. Two regions with more than 90% identity can be identified within the murine and human CD28 polypeptide sequences. One region is in the cytoplasmic domain (amino acids 170 to 199) and it is possible that this region plays a role in CD28-mediated signal transduction

TABLE 20. *Failure of cyclosporine A to inhibit anti-CD28-induced T cell activation*[a]

Stimulus	[^{3}H]TdR incorporation (CPM × 10^{-3})		
	Media	Cyclosporine A	Percent inhibition
Anti-CD3	77	10	90
Anti-CD3 + PMA	145	55	62
Anti-CD28 + PMA	111	112	0

Adapted from ref. 172.

[a] $CD28^+$ T cells were cultured with solid-phase anti-CD3 in the presence or absence of PMA or with soluble anti-CD28 and PMA. Cyclosporine A was added at a final concentration of 800 ng/ml.

contrast to activation via all the other accessory molecules described in this chapter.

The mechanism for induction and augmentation of T cell activation by engagement of the CD28 antigen has been analyzed at the molecular level. Culture of T cells with anti-CD28 mAb alone does not induce lymphokine gene expression in resting T cells. However, the CD28 activation pathway significantly enhances the production of multiple lymphokines including IL-2, IFN-γ, TNF-α, lymphotoxin, and GM-CSF by anti-CD3 or PMA activated human T cells (174). Anti-CD28 can even produce an approximately 10-fold enhancement of IL-2 gene expression in T cells stimulated by optimal concentrations of PMA and ionomycin. One mechanism proposed for this CD28-mediated enhancement of lymphokine gene expression is stabilization of cytokine mRNA (175). A second mechanism for the induction of cytokine gene expression via CD28 involves the formation of a protein complex that binds to a site on the IL-2 gene between -164 and -154 base pairs from the transcription start site and increases IL-2 enhancer activity fivefold (176). The region of the IL-2 enhancer that responds to CD28 stimulation is absolutely conserved between human and murine IL-2 genes and similar sequences are present in the 5′ flanking regions of several other lymphokine genes. It has yet to be resolved whether CD28-mediated enhancement of cytokine gene expression is primarily secondary to mRNA stabilization or to the induction of transcription by this unique DNA binding complex.

Receptor–Ligand Interactions

In addition to the stimulatory capacity of anti-CD28 mAbs on resting T cells, anti-CD28 mAbs have also been reported to inhibit certain T cell responses such as the mixed lymphocyte reaction or the activation of antigen-specific T cell clones. Taken together, these studies raised the possibility that anti-CD28 mAbs were either mimicking or blocking the interaction of CD28 with its physiological ligand. A role for CD28 in cell adhesion was directly demonstrated by showing that the CD28 antigen, following expression at high levels in CHO cells, mediated specific intercellular adhesion with human lymphoblastoid cells lines, leukemic B cells, and activated normal B cells. CD28 adhesion was not dependent on divalent cations and was specifically inhibited by mAbs to the B cell activation antigen B7 (177). Transfected COS cells, which expressed B7, would specifically adhere to CD28 transfected CHO cells and this adhesion could be blocked by mAbs to both CD28 and B7. Since both CD28 and B7 are found at relatively low levels on resting lymphoid cells, CD28–B7-mediated adhesion may play a major role in maintaining or amplifying the immune response rather than initiating it.

The importance of the CD28–B7 pair in mediating cell–cell interaction was further elucidated by using fusion proteins composed of the extracellular domains of B7 or of CD28 and the hinge-CH2-CH3 domains of human IgG1 (178). The apparent K_D for the interaction of B7 and CD28 was determined to be approximately 200 nM, which is similar to the affinities reported for mAbs and the affinities estimated for other lymphoid cell adhesion molecules. Because of the close structural similarity between CD28 and CTLA-4, a soluble CTLA-4 Ig fusion protein was constructed and also bound to the surface of B7 transfected CHO cells. The K_D of binding of soluble B7 Ig for immobilized CTLA-4 Ig was estimated to be about 12 nM. Thus CTLA-4 Ig is also a receptor for the B cell activation antigen B7. The avidity of B7 Ig for immobilized CTLA-4 Ig was found to be about 20-fold greater than its avidity under identical conditions for immobilized CD28 Ig. The functional significance of the binding of CTLA-4 Ig to B7 is at present unknown. Presumably, because CD28 and CTLA-4 have homologous cytoplasmic domains, it is likely that CTLA-4 Ig may have signaling functions similar to CD28.

B7 transfected CHO cells have been shown to be effective costimulators of both murine and human T cell proliferation induced by immobilized anti-CD3 (Table 21),

TABLE 21. *Costimulatory effects of B7 on anti-CD3-induced T cell activation*[a]

Costimulus	[^{3}H]TdR incorporation (CPM × 10^{-3})
None	26
B7 Ig	175
CHO, $B7^+$	114
CHO $B7^-$	22
Anti-CD28	156

Adapted from ref. 178.

[a] Human lymphocytes were cultured in the presence of solid-phase anti-CD3 and the indicated costimulus. The B7 Ig fusion protein, B7 transfected CHO cells, and anti-CD28 mAb all provided potent costimulatory activity.

CORPORATE
TRANSLATIONS, INC.

CERTIFICATION

This is to certify that Corporate Translations, Inc. has performed a true translation for ***Rhône-Poulenc Rorer*** of ***French application FR95/01662, Medicinal Association Useful for the In Vivo Transfection and Expression of Exogenes, filed February 14, 1995 in French***. This document was prepared by a translator who is fully bilingual in both French and English.

Authorized Signature:

Mary C. Gawlicki
President
Corporate Translations, Inc.

January 6, 2000

"Subscribed and sworn to before me this 6th day of January, 2000"

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Date Commission Expires: 11/30/02

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CERTIFICATE OF UTILITY – CERTIFICATE OF ADDITION

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Paris, December 10, 1999

For the Director General of the
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The Head of the Patents Department

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REQUEST
FOR DELIVERY OF A CERTIFICATE OF INDUSTRIAL PROPERTY*

1

a X PATENT OF INVENTION
b CERTIFICATE OF UTILITY
c DIVISIONAL APPLICATION
d CONVERSION OF A EUROPEAN PATENT OF INVENTION

For c and d, specify: Type, No. and date of initial application

2 MANDATORY OPTIONS when filing (except for certificate of utility)

THE APPLICANT REQUESTS **POSTPONEMENT** OF THE SEARCH REPORT — YES / X NO

IF THE OPTION SELECTED IS NO AND IF THE APPLICANT IS AN INDIVIDUAL, HE REQUESTS STAGGERED PAYMENT OF THE SEARCH REPORT FEE — YES / X NO

TYPE | NUMBER | DATE OF INITIAL APPLICATION

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7 TITLE OF THE INVENTION

MEDICINAL ASSOCIATION USEFUL FOR THE *IN VIVO* TRANSFECTION AND EXPRESSION OF EXOGENES

8 APPLICANT(S): First and last names (underline last name) or name and legal form

SIREN No.

CNRS: NATIONAL CENTER FOR SCIENTIFIC RESEARCH
IGR: INSTITUT GUSTAVE ROUSSY
INSERM: NATIONAL INSTITUTE OF HEALTH AND MEDICAL RESEARCH

9 COMPLETE ADDRESS(ES)	COUNTRY
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10 NATIONALITY(IES)
French

11 INVENTOR(S)
THE APPLICANT IS THE SOLE INVENTOR — YES / NO
If the answer is no, see explanatory note

12 IF THE APPLICANT IS A NON-ASSESSABLE INDIVIDUAL, HE REQUESTS* OR HAS REQUESTED REDUCTION OF THE FEES — YES / NO

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X SEARCH REPORT
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X CLAIM (starting from 11a)

13 DECLARATION OF **PRIORITY** OR REQUEST FOR **SURPLUS FROM FILING DATE** OF A PREVIOUS APPLICATION	COUNTRY OF ORIGIN	FILING DATE	NUMBER

14 DIVISIONS	PRIOR TO THE PRESENT APPLICATION No.	No.	No.	No.

15 SIGNATURE OF APPLICANT OR AGENT AND NAME AND POSITION OF SIGNATORY – REGISTRATION No. [signature]	SIGNATURE OF RECEPTION ATTENDANT	SIGNATURE AFTER REGISTRATION OF APPLICATION AT THE NIIP [signature]
Pascale LE COUPANEC		

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Patent Administration Division

NAME OF THE INVENTOR (if the applicant is not the inventor or sole inventor	National Registration No. 9501662

Title of the Invention:

MEDICINAL ASSOCIATION USEFUL FOR THE *IN VIVO* TRANSFECTION AND EXPRESSION OF EXOGENES

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NOTE: In exceptional cases, the name of the inventor can be followed by that of the company to which he belongs (company that employs him) when the latter is different from the applicant or proprietary company.

Date and signature(s) of the applicant(s) or agent

Antony, February 14, 1995
[signature]

Pascale LE COUPANEC

BA 113/160392

DOCUMENT INCLUDING CHANGES

PAGE(S) OF THE DESCRIPTION OR CLAIMS OR PLATE(S) OF DRAWING			MC*	DATE OF CORRESPONDENCE	DATE STAMP OF EXAMINER
Modified	Deleted	Added			
24				25 APRIL 1995	[illegible] 04 MAY 1995 [poorly legible]

Any change in the wording of the original claims, unless it follows from the provisions of Article 28 of the Decree of September 19, 1979, is indicated by the notation "MC" (modified claims).

The present invention pertains to the field of gene therapy and notably to the use of adenoviruses for the expression of genes of therapeutic interest. More particularly, it pertains to a new method of treating diseases of genetic origin based on the combined use of two types of therapeutic agents.

Gene therapy consists of correcting a deficiency or an anomaly (mutation, aberrant expression, etc.) by introducing genetic information into the affected cell or organ. This genetic information can be introduced either *in vitro* or *ex vivo* into a cell extracted from the organ, with the modified cell then being reintroduced into the organism, or directly *in vivo* into the appropriate tissue. In the second case, there are different physical transfection techniques, including the use of viruses as vectors. In that regard, different viruses have been tested for their capacity to infect certain cell populations. In particular, retroviruses (RSV [respiratory syncytial virus], HMS, MMS, etc.), the HSV [herpes simplex virus] viruses, adeno-associated viruses and adenoviruses.

Among these viruses, the adenoviruses have certain properties that are advantageous for use in gene therapy. They have a fairly broad host spectrum, they are capable of infecting quiescent cells and they do not combine with the genome of the infected cell. Adenoviruses are viruses with linear double strand DNA of approximately 36 kb. Notably, their genome comprises a repetitive inverse sequence (ITR) at the end, an encapsidation sequence, early genes and late genes (cf Figure 1). The principal early genes are the E1 (E1a and E1b), E2, E3 and E4 genes. The principal late genes are the L1 to L5 genes.

Considering the properties of the aforementioned adenoviruses, the latter have already been used for gene transfer *in vivo*. In that regard, different vectors derived from adenoviruses have been prepared, incorporating different genes (ß-gal, OTC, α-1AT, cytokines, etc.). In each of these constructions, the adenovirus was modified to render it incapable of replication in the infected cell. Thus, the constructions described in the prior art are adenoviruses with E1 regions (E1a and/or E1b), and possibly E3 deleted, and into which a heterologous DNA sequence is inserted (Levrero et al., Gene 101 (1991) 195; Gosh-Choudhury et al., Gene 50 (1986) 161).

However, as for all known viruses, the administration of a wild-type (Routes et al., J. Virol. 65 (1991) 1450) or defective recombinant adenovirus for replication (Yang et al., PNAS (1994) 4407), induced an important immune response.

In fact, one of the major roles of the immune system is to destroy the non-self or self-altered elements in the body. To do this, the immune system develops two types of defensive action. The first consists in producing antibodies which will bind to the antigen in question in order either to inactivate it or to organize its removal from the body. In this case, we speak of humoral immunity. The second method of defense, which is called cellular immunity, makes use of cytotoxic T cells. These cells specifically attack the infected cells, that is, those that have acquired antigens on their surface. More precisely, the receptors of cytotoxic T cells recognize the antigens introduced in association with molecules of the class I major histocompatibility complex (MHC-I) on the surface of infected cells. This is followed by the destruction of the infected cell.

Consequently, this immune response developed against infected cells constitutes a major obstacle to the use of viral vectors in gene therapy since (i) by inducing the destruction of the infected cells, it limits the duration of expression of the therapeutic gene, and therefore, the therapeutic effect; (ii) at the same time, it induces an important inflammatory response, and (iii) it leads to the rapid elimination of the infected cells after repeated injections. It is understood that the amplitude of this immune response against infected cells varies according to the nature of the organ undergoing the injection and the injection method used. Thus, the expression of the ß-galactosidase, which is encoded by a recombinant adenovirus administered into the muscle of immunocompetent mice is reduced to minimum levels 40 days after the injection (Kass-Eisler et al., PNAS 90 (1993) 11498). Moreover, the expression of transfected genes by adenoviruses in the liver is significantly reduced within 10 days following the injection (Yang Y et al. 1994 Immunity 1 433-442), and the expression of factor IX which was transferred by the adenovirus into the hepatocytes of hemophilic dogs disappeared 100 days after the injection (Kay et al., PNAS 91 (1994) 2353).

From the standpoint of the use of vectors derived from adenoviruses in gene therapy, therefore, it seems to be necessary to control the immune response developed in opposition to them or against the cells that they infect.

From the preceding information, it is clear that activation of the immune system requires, first of all, recognition by the latter of the non-self or self-altered elements, such as for example, vectors derived from adenoviruses, which must be destroyed. A tolerance phenomenon arises from the recognition of self or non-self.

It is precisely at this level that the present invention intervenes. It is aimed at preventing the rapid elimination of adenoviruses from infected cells, and therefore, consequently prolonging the *in vivo* expression of the therapeutic gene that they deliver.

Recently, the Applicant has demonstrated that the co-expression of certain genes in the infected cells can induce an immunoprotective effect, thus, allowing the vectors and/or the infected cells to be released into the immune system. [The applicant] has notably developed adenoviruses in which the expression of a gene of therapeutic interest is coupled with that of an immunoprotective gene (FR No. 94 12346). Notably, this may be a gene whose product acts on the activity of the major histocompatibility complex (MHC) or on the activity of cytokines, thus permitting it to reduce considerably, or even to eliminate any immune reaction against the vector or the infected cells. They at least partially inhibit the expression of MHC proteins or the introduction of antigens with the advantageous result of a notable reduction in the immune reaction against the vector or the infected cells, and thus, a prolonged therapeutic effect.

The applicant has unexpectedly demonstrated that it was possible to prolong significantly over time the therapeutic effect of such a vector by associating it with an immunosuppressant. The destruction, by the immune system, of the vector considered or of the infected cells is delayed over time for a clearly longer period than that which could be expected from the simple juxtaposition of the immunoprotective effects induced by said vector and immunosuppressive agent, respectively. Advantageously, the drug association that is the subject of this invention induces a pseudo-inertia phenomenon of the immune system which is favorable to the prolonged expression over time of a therapeutic gene.

According to the invention, an immunosuppressive agent is any compound that is capable of partially or completely inhibiting at least one immune signaling pathway. In general, immunosuppressive agents are compounds that are classically administered following an organ transplantation in order to prevent any reaction rejecting the latter. The compounds that are classically used are either chemical agents, such as cyclosporin, FK506, azathioprine and corticosteroids, or monoclonal or polyclonal antibodies. With regard to the first category of immunosuppressive agents, their function is to prevent the synthesis of interluekin-2 and/or other lymphokines which play an important role in the growth and activity of lymphocytes. Unfortunately, the efficacy of this type of immunosuppressive agent requires continuous administration, which is known to have more or less long-term harmful toxic effects. Thus, azathioprine is a known bone marrow suppressant and cyclosporin is nephrotoxic and may also cause hypertension and neurological problems.

More particularly, with regard to the antibodies, they are antibodies directed against the lymphoid cells of the immune system. The primary antibody used as an immunosuppressive agent is anti-CD3, directed against T lymphocytes. Its target is one of the CD3 molecules that make up the T-cell receptor-antigen complex. The result is the inactivation of the immune cells carrying that molecule thus blocking activation of the immune system. Therefore, it would be incapable of reacting to the presence of infected cells, even though antigens are present. On the same principle, it is possible to make use of anti-CD4, -CD2, -CD8, -CD28, -B7, -ICAM-1 and –LFA1 antibodies.

The applicant has now developed a new method of treatment, which is particularly effective in significantly delaying or even inhibiting the immune system reaction without raising the problem of toxicity.

More precisely, the present invention derives from the demonstration of a particularly important synergistic effect related to the combined use of a recombinant adenovirus in which the expression of a gene of therapeutic interest is coupled with that of an immunoprotective gene, as described above, and at least an immunosuppressive agent.

A first objective of this invention, therefore, pertains to a medicinal association of at least an immunosuppressive agent and at least a recombinant adenovirus the genome of which includes a first

recombinant DNA containing a therapeutic gene and a second recombinant DNA containing an immunoprotective gene, for consecutive, intermittent and/or simultaneous use over time, which is useful for *in vivo* and/or *ex vivo* exogenic transfections.

As indicated above, the invention is based, notably, on the demonstration of a synergistic effect between the activity of the immunosuppressive agent and the effect of the immunoprotective gene expressed on the expression of the therapeutic gene.

This combined use permits a clearly prolonged therapeutic effect and advantageously requires significantly lower doses, notably of the immunosuppressive agent.

As indicated below, the two components of the combined treatment of the present invention can be used consecutively, intermittently and/or simultaneously over time. Preferably, the immunosuppressive agent is injected before and after the injection of the adenovirus. According to this mode of implementation of the present invention, the administration of the immunosuppressive agent can be spaced over time, and more preferably, repeated at regular intervals. In this particular case, the two components are packaged separately. In the case of simultaneous administration, they can be mixed extemporaneously before being administered together, or on the other hand, administered simultaneously, but in a separate manner. In particular, the routes of administration of the two agents can be different.

According to the present invention, it is possible to use as the immunosuppressive agent any compound that is capable of partially or completely inhibiting at least one immune signaling pathway. In particular, it can be selected from cyclosporin, FK506, azathioprine, corticosteroids and any monoclonal or polyclonal antibody. These are preferably antibodies capable of inactivating the immune molecules or of causing the destruction of the immune cells carrying these molecules. Notably, it is possible to use as antibodies, anti-CD4, -CD3, CD2, -CD8, -CD28, -B7, -ICAM-1, -LFA-1. It is also possible to use hybrid molecules such as CTLA4Ig, a fusion protein between the CTLA-4 molecule (a CD28 homolog) and an immunoglobulin. The GlFc site of this molecule, by binding to the B7 molecule, is known to be capable of inhibiting the activation of T cells (D.J. Lenschow; Science, 257, 789, 1992). It is clear that the scope of the present invention is in no way limited to the immunosuppressive agents enumerated above. These immunosuppressive agents can be used in isolated form or combined.

With regard to the recombinant DNAs present in the genome of the adenovirus used according to the present invention, they are DNA fragments that contain the gene in question (therapeutic or immunoprotective) and possibly signals that permit their expression, constructed *in vitro* and then inserted into the genome of the adenovirus. The recombinant DNA used as part of the present invention can be complementary DNA (cDNA), genomic DNA (gDNA) or hybrid constructions consisting, for example, of a cDNA into which one or more introns would be inserted. It may also involve synthetic or semisynthetic sequences. These DNAs can be of human, animal, plant, bacterial, or viral, etc. origin. The use of cDNA or gDNA is particularly advantageous.

As the therapeutic gene that can be used for the construction of the vectors of the present invention, it is possible to cite any gene encoding for a product that has a therapeutic effect. The coded product can be a protein, a peptide, an RNA, etc.

If it is a protein product, it can be homologous to the target cell (that is, a product that is normally expressed in the target cell when the latter presents no pathology). In this case, the expression of a protein, for example, makes it possible to overcome insufficient expression in the cell or the expression of an inactive or weakly active protein because of a modification or even to over-express said protein. The therapeutic gene can also encode for a mutant of a cellular protein, which has increased stability, modified activity, etc. The protein product can also be heterologous to the target cell. In this case, a protein expressed, for example, to complete or to provide activity that is lacking in the cell, permitting it to combat a pathology or to stimulate an immune response.

Among the therapeutic protein products in the terms of the present invention, it is possible to cite, more particularly, enzymes, blood derivatives, hormones, interleukins, interferons, TNF [tumor necrosing factor], etc. (Fr 92 03120), growth factors, neurotransmitters or their precursors or synthesis enzymes, trophic factors: BDNF, CNTF, NGF [nerve growth factor], IGF [insulin-like growth factor], GMF, aFGF [fibroblast growth factor], bFGF, NT3, NT5, HARP/pleiotropin, etc.; apolipoproteins: ApoAI, ApoAIV, ApoE, etc. (Fr 93 05125), dystrophin or a mini-dystrophin (Fr 91 11947), the CFTR [Cystic Fibrosis Transmembrane Regulator] protein which is associated with cystic fibrosis, tumor suppressive genes:

p53, Rb, Rap1A, DCC, k-rev, etc. (Fr 93 04745), genes encoding for factors involved in coagulation: Factors VII, VIII, IX, genes intervening in DNA repair, etc.

As indicated above, the therapeutic gene can also be an antisense gene or sequence, and its expression in the target cell controls the expression of genes or the transcription of cellular mRNA. Such sequences can, for example, be transcribed in the target cell by complementary RNA of cellular mRNA, and thus block their translation to protein, according to the technique described in EP patent 140 308. The antisense [sequences] also include sequences coding for ribozymes, which are capable of selectively destroying the target RNAs (EP 321 201).

Therapeutic genes can be of human, animal, plant, bacterial or viral, etc. origin. They can be obtained by any technique know to those skilled in the art, and notably, by screening libraries, by chemical synthesis, or even by combined methods including the chemical or enzymatic modification of sequences obtained by library screening .

Different types of immunoprotective genes can be used within the scope of the present invention. As explained previously, this is a gene whose product acts on the activity of the major histocompatibility complex (MHC) or on the activity of cytokines. It is preferably a gene whose product at least partially inhibits the expression of the MHC proteins or the introduction of antigens. Certain genes contained in the E3 region of the adenovirus, the ICP47 gene of the herpes virus, or the UL18 gene of the cytomegalovirus can be cited as preferred examples.

The E3 region of the adenovirus genome contains different reading phases which, by alternative splicing give rise to different proteins. Among the latter, protein Gp19k (or E3-19k) is a glycosylated transmembrane protein located in the membrane of the endoplasmic reticulum (ER). This protein includes a luminal field binding the MHC-I molecules and a C-terminal cytoplasmic end capable of binding the microtubules (or tubulin) which act to anchor protein gp19k in the ER membrane. Gp19k is thus capable of preventing the expression of MHC-I molecules on the surface of the cells by interaction and sequestration at the level of the ER. However, in the absence of viral replication, protein gp12k is weakly expressed by the adenovirus. Moreover, the expression of gp19k is also dependent on splicing. The introduction into the vectors of the invention of a recombinant DNA containing a sequence

(preferably cDNA) encoding for gp19k makes it possible to control and to optimize the expression of said protein. In particular, the use of constitutive promoters and the suppression of other reading phases make it possible to increase greatly the expression of this protein and to free it from dependence on viral replication and the presence of inducing elements. This is particularly advantageous in considerably reducing lysis of infected cells by CTLs [cytotoxic T-lymphocytes], thus increasing and prolonging the *in vivo* production of the therapeutic gene.

Other proteins encoded by the E3 region of the genome of the adenovirus such as proteins 10,4k and 14,5k represent certain advantageous properties in view of their incorporation in the vectors of the invention.

The ICP47 gene of the herpes simplex virus constitutes another immunoprotective gene that is particularly advantageous in terms of the present invention. Cells infected by the herpes simplex virus are resistant to the lysis induced by the CTLs. It has been demonstrated that this resistance might be conferred by the ICP47 gene, which is capable of reducing the expression of MHC –I molecules on the surface of the cells. The incorporation of the ICP47 gene in a recombinant DNA according to the invention also permits the recombinant viruses of the invention to escape the immune system.

The UL18 gene of the cytomegalovirus is another preferred example of the immunoprotective gene according to the invention. The product of the UL18 gene is capable of binding ß2-microglobulin (Browne et al. Nature 347 (1990) 7707). The ß2-microglobulin is one of the chains of MHC-I molecules. The incorporation of the UL18 gene into a recombinant DNA according to the invention thus makes it possible to reduce the number of functional ß2-microglobulin molecules in the cells infected by the viruses of the invention, and thus to diminish the capacities of these cells to produce complete and functional MHC-I molecules. This type of construction, therefore, protects the infected cells from lysis by CTLs.

As indicated above, the immunoprotective gene used as part of the present invention is, in another preferred mode of embodiment, a gene whose product inhibits the activity of the signaling pathways of cytokines. Cytokines are a family of secreted proteins which act as signaling molecules for the immune

system. They can attract immune cells, activate them, and induce their proliferation, and they can even act directly on the infected cells to kill them.

Among the genes whose product affects the activity or signaling pathways of cytokines, genes that are involved in the synthesis of cytokines, or those whose product is capable of sequestering cytokines, of antagonizing their activity or interfering with intercellular signaling pathways can be cited. As preferred examples, in particular the BCRF1 gene of the Epstein Barr virus, the crmA and crmB genes of the cowpox virus, the B15R and B18R genes of the vaccinia virus, the US28 gene of the cytomegalovirus, and the E3-14,7, E3-10,4 and E3-14,5 genes of the adenovirus can be cited.

The B15R gene of the vaccinia virus encodes for a soluble protein that is capable of binding interleukin-1ß (the secreted form of interleukin-1), and thus preventing this cytokine from binding to its cellular receptors. Interleukin-1 is actually one of the first cytokines produced in response to antigenic aggression, and it plays a very important role in the signaling of the immune system at the beginning of the infection. The possibility of incorporating the B15R gene into a vector according to the invention advantageously makes it possible to reduce the activity if IL-1ß, notably on the activation of the immune cells, and therefore, to locally protect the cells infected by the viruses of the invention against an important immune response. Genes that are homologous to the B15R gene can also be used, such as the ---gene of the cowpox virus.

In the same manner, the B18R gene of the vaccinia virus encodes for a protein that is homologous to the interleukin-6 receptor. This gene, or any functional homolog, can also be used in the vectors of the invention to inhibit interleukin-6 from binding to its cellular receptor, and thus to reduce the immune response locally.

Again, in the same manner, the crmB gene of the cowpox virus can advantageously be used. This gene actually encodes for a secreted protein that is capable of binding TNF and of entering into competition with the TNF receptors on the cell surface. Therefore, in the viruses of the invention, this gene makes it possible to reduce locally the concentration of active TNF that can destroy the infected cells. Other genes encoding for proteins that are capable of binding TNF and at least partially inhibiting it from binding to its receptors can also be used.

The crmA gene of the cowpox virus encodes for a protein that has activity that inhibits serine- type proteases, and which is capable of inhibiting interleukin-1ß synthesis. This gene can be used, therefore, to reduce the local concentration of interleukin-1, and thus to reduce the development of the immune and inflammatory response.

The BCRF1 gene of the Epstein Barr virus encodes for an analog of interleukin 10. The product of this gene is a cytokine that is capable of reducing the immune response and changing its specificity, while inducing the proliferation of B lymphocytes.

The US28 gene of the cytomegalovirus encodes for a protein that is homologous to the 1α-macrophage inflammatory protein (MIP-1α). Therefore, this protein is capable of acting as a competitor of MIP receptors, and thus of inhibiting their activity locally.

The product of genes E3-14,7, E3-10,4 and E3-14,5 of the adenovirus is capable of blocking the transmission of the intercellular signal mediated by certain cytokines. When the cytokines bind to their receptor on the surface of an infected cell, a signal is transmitted to the nucleus to induce cell death or to stop protein synthesis. In particular, this is the case with tumor necrosis factor (TNF). The incorporation of genes E3-14,7, E3-10,4 and/or E3-14,5 into a recombinant DNA according to the invention in view of their constitutive or regulated expression makes it possible to block the intercellular signaling induced by the TNF, and thus to protect the cells infected by the recombinant viruses of the invention from the toxic effects of this cytokine.

Local and transient inhibition can be particularly advantageous. It can be obtained notably by the choice of special expression signals (cytokine-dependent promoters, for example) as indicated below.

It is understood that other homologous genes or those having similar functional properties can be used for the construction of the vectors of the invention. These different genes can be obtained by any technique known to those skilled in the art, and notably by library screening, by chemical synthesis, or even by combined methods that include chemical or enzymatic modification of the sequences obtained by library screening. Moreover, these different genes can be used alone or in combination(s).

The insertion of the genes in question in the form of recombinant DNA according to the invention offers greater flexibility in the construction of adenoviruses, and permits better control of the expression of said genes.

Thus, the recombinant DNAs (and therefore, the genes of interest) incorporated into adenoviral vectors according to the present invention can be organized in different ways.

First of all, they can be inserted into the same site of the adenovirus genome, or different, selected sites. In particular, recombinant DNA can be inserted at least partly in the E1, E3 and/or E4 regions of the adenovirus genome, replacing or supplementing viral sequences.

Preferably, recombinant DNA is inserted, at least partly, in the E1, E3 or E4 regions of the adenovirus genome. When it is inserted at two different sites, within the scope of the invention, it is preferred to use regions E1 and E3 or E1 and E4. The examples actually show that this organization permits a high expression of the two genes, without interference between the two. Advantageously, the recombinant DNAs are inserted by replacing viral sequences.

Each recombinant DNA can then include an identical or different transcriptional promoter. This configuration makes it possible to obtain higher levels of expression and to offer better control of the expression of the genes. In this case, the two genes can be inserted in the same orientation or in opposite orientations.

They can also constitute an unique transcriptional entity. In this configuration, two recombinant DNAs are contiguous and positioned such that the two genes are under the control of a single promoter, and give rise to a unique premessenger RNA. This arrangement is advantageous since it uses a single transcriptional promoter.

Finally, the use of recombinant DNA according to the invention makes it possible to use different types of transcriptional promoters, and notably, promoters that are strong or weak, regulated or constitutive, tissue-specific or ubiquitous, etc.

The choice of expression signals and of the respective position of the recombinant DNA is particularly important in obtaining a high expression of the therapeutic gene and an important immunoprotective effect.

A particularly preferred embodiment of the present invention makes use of a defective adenovirus comprising a first recombinant DNA containing a therapeutic gene and a second recombinant DNA containing an immunoprotective gene, in which the two recombinant DNAs are inserted in the E1 region.

A particularly preferred embodiment of the present invention uses a defective adenovirus comprising a first recombinant DNA containing a therapeutic gene, inserted in the E1 region, and a second recombinant DNA containing an immunoprotective gene inserted in the E3 region.

As indicated above, the adenoviruses of the present invention are defective, that is, they are incapable of replicating autonomously in the target cell. Generally, therefore, the genome of defective adenoviruses according to the present invention lacks at least the sequences necessary for the replication of said virus in the infected cell. These regions can either be eliminated (completely or partly), or rendered non-functional, or substituted by other sequences, and notably by therapeutic genes. The defective character of the adenoviruses of the invention is an important element since it ensures non-dissemination of the vectors of the invention after administration.

In a preferred method of embodiment, the adenoviruses of the invention comprise ITR sequences and a sequence that permits encapsidation, and they possess a deletion of all or part of the E1 gene.

Repeated inverse sequences (ITR) are the source of adenovirus replication. They are located at the 3' and 5' ends of the viral genome (cf Figure 1), where they can easily be isolated according to classic molecular biology techniques known to those skilled in the art. The nucleotide sequence of the ITR sequences of human adenoviruses (in particular, serotypes Ad2 and Ad5) is described in the literature, as well as that of canine adenoviruses (notably, CAV1 and CAV2). With regard to adenovirus Ad5, for example, the left ITR sequence corresponds to the region that includes nucleotides 1 to 103 of the genome.

The encapsidation sequence (also called the Psi sequence) is necessary for the encapsidation of viral DNA. This region must be present to permit the preparation of the defective recombinant adenoviruses according to the invention. The encapsidation sequence is located in the genome of the adenoviruses, between the left ITR (5') and the E1 gene (Cf Figure 1). It can be isolated or synthesized artificially by classic molecular biology techniques. The nucleotide sequence of the encapsidation sequence of human adenoviruses (in particular, serotypes Ad2 and Ad5) is described in the literature, as well as that of canine adenoviruses (notably CAV1 and CAV2). With regard to the Ad5 adenovirus, for example, the encapsidation sequence corresponds to the region comprising nucleotides 194 to 358 of the genome.

More preferably, the adenoviruses of the invention comprise the ITR sequences and a sequence that permits encapsidation and they have a deletion of all or part of the E1 and E4 genes.

In a particularly preferable embodiment according to the invention, all or part of the E1, E3 and E4 genes is deleted from the genome of the adenovirus, and even more preferably, all or part of genes E1, E3, L5 and E4.

The adenoviruses of the invention can be prepared from adenoviruses of different origins. In fact, there are different types of adenovirus serotypes, in which the structure and properties vary somewhat, but which have a comparable genetic organization. Thus, the information described in the present application can easily be reproduced by those skilled in the art for any type of adenovirus.

More particularly, the adenoviruses of the invention can be of human, animal or combined (human and animal) origin.

With regard to adenoviruses of human origin, it is preferable to use those classified in group C. More preferably, among the different human adenovirus serotypes, within the context of the present invention, it is preferable to use type 2 or 5 (Ad2 or Ad5) adenoviruses.

As indicated above, the adenoviruses of the invention can also be of animal origin, or can include sequences from adenoviruses of animal origin. In fact, the applicant has demonstrated that adenoviruses of animal origin are extremely efficacious in infecting human cells, and that they are incapable of propagating in the human cells in which they have been tested (Cf Application FR 93 05954). The

applicant has also demonstrated that adenoviruses of animal origin are in no way trans-complemented by adenoviruses of human origin, which eliminates any risk of recombination and propagation *in vivo*, in the presence of a human adenovirus, which might lead to the formation of an infectious particle. The use of adenoviruses or of adenovirus regions of animal origin is, therefore, particularly advantageous since the risks inherent in the use of viruses as vectors in gene therapy are even lower.

Adenoviruses of animal origin that can be used within the context of the present invention can be of canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or even simian (example: SAV)origin. More particularly, among the avian adenoviruses, ATCC accessible serotypes 1 to 10 , such as for example, Phelps (ATCC VR-432), Fontes (ATCC VR-280), P7-A (ATCC VR-827), IBH-2A (ATCC VR-828), J2-A (ATCC VR-829), T8-A (ATCC VR-830), K-11 (ATCC VR-921), and also ATCC referenced strains VR-831 to 835 can be cited. Among the bovine adenoviruses, different known serotypes can be used, and notably those accessible at the ATCC (types 1-8) under references ATCC VR-313, 314, 639-642, 768 and 769. The following murine adenoviruses can also be cited: FL (ATCC VR-550) and E20308 (ATCC VR-528), ovine adenovirus type 5 (ATCC VR-1343), or type 6 (ATCC VR-1340); porcine adenovirus 5359) [sic], or simian adenoviruses such as, in particular, ATCC adenoviruses referenced as numbers VR-591-594, 941-943, 195-203, etc.

Preferably, among the different adenoviruses of animal origin, within the context of the invention, adenoviruses or adenovirus regions of canine origin are used, and particularly all strains of CAV2 adenoviruses [Manhattan strain or A26/61 (ATCC VR-800), for example]. Canine adenoviruses have been the subject of several structural studies. Thus, complete restriction maps of the CAV1 and CAV2 adenoviruses have been described in the prior art (Spibey et al., J. Gen. Virol. 70 (1989) 165), and E1a, E3 genes, as well as ITR sequences have been cloned and sequenced (see, in particular, Spibey et al., Virus Res. 14 (1989) 241; Linné, Virus Res. 23 (1992) 199, WO 91/11525).

The defective recombinant adenoviruses according to the invention can be prepared in different ways.

A first method consists of transfecting the DNA from the defective recombinant virus prepared *in vitro* (either by splicing or in plasmid form) into a competent cell line, that is, one that has all of the functions necessary for complementation of the defective virus in the *trans* configuration. These functions are preferably integrated into the cell's genome, which makes it possible to avoid the risks of recombination, and provides the cell line with increased stability.

A second approach consists of cotransfecting into an appropriate cell line the DNA of the defective recombinant virus prepared *in vitro* (either by splicing, or in plasmid form) and the DNA of a helper virus. According to this method, it is not necessary to have a competent cell line capable of complementing all of the defective functions of the recombinant virus. Some of these functions are, in fact, complemented by the helper virus. This helper virus must itself be defective and the cell line has the functions that are necessary for its complementation in the *trans* configuration. Among the cell lines that can be used within the scope of this second approach, the human embryo kidney line 293, KB [human oral epidermoid carcinoma] cells, HeLa cells, MDCK [Madin-Darby canine kidney], GHK, etc. can be cited (Cf examples).

Next, the vectors which are multiplied are recovered, purified and amplified according to classic molecular biology techniques.

According to one embodiment variation, it is possible to prepare *in vitro*, either by splicing or in plasmid form, the DNA of the defective recombinant virus carrying the appropriate deletions and the two recombinant DNAs. As indicated above, the vectors of the invention advantageously possess a deletion of all or part of certain viral genes, notably genes E1, E3, E4 and/or L5. This deletion may correspond to any type of suppression affecting the gene in question. It may, in particular, involve suppression of all or part of the encoding region of said gene, and/or all or part of the transcription promoting region of said gene. Suppression generally takes place on the DNA of the defective recombinant virus, for example, by digestion by the appropriate restriction enzymes, then splicing, according to molecular biology techniques, as illustrated in the examples. The recombinant DNA can then be inserted into that DNA by enzyme cleavage then splicing, in the selected regions and in the chosen orientation.

DNA obtained in this manner, which therefore carries the appropriate deletions and the two recombinant DNAs, makes possible direct generation of the defective recombinant adenovirus carrying said deletions and recombinant DNA. This first variation is particularly well-suited to the embodiment

of the recombinant adenoviruses in which the genes are arranged in the form of a unique transcriptional unit, or under the control of separate promoters, but inserted into a single site on the genome.

It is also possible to prepare the recombinant virus in two steps, permitting the successive introduction of two recombinant DNAs. Thus, the DNA from a first recombinant virus carrying the appropriate deletions (or some of said deletions) and one of the recombinant DNAs is constructed, by splicing or in plasmid form. This DNA is then used to generate a first recombinant virus carrying said deletions and a recombinant DNA. The DNA from this first virus is then isolated and cotransfected with a second plasmid or the DNA from a second defective recombinant virus carrying the second recombinant DNA, the appropriate deletions (the part that is not present on the first virus), and a region that permits homologous recombination. This second step thus generates the defective recombinant virus carrying the two recombinants DNAs. This preparation variant is particularly appropriate for the preparation of recombinant viruses carrying two recombinant DNAs inserted in two different regions of the adenovirus genome.

The two agents according to the invention, namely the immunosuppressive agent and the recombinant adenovirus can be formulated with a view to topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, etc. administration.

Preferably, the respective pharmaceutical formulation or formulations contain vehicles that are pharmaceutically acceptable for an injectable formulation. In particular, they can be sterile, isotonic saline solutions (monosodium phosphate, disodium phosphate, sodium, potassium, calcium or magnesium, etc. chloride, or mixtures of these salts), or dry compositions, notably freeze dried, which by adding sterilized water or physiological serum, according to the case, permits the constitution of injectable solutions.

The doses of immunosuppressive agent and adenoviruses used for the injection can be adjusted as a function of different parameters, and notably, as a function of the method of administration used, the pathology in question, the gene to be expressed, or even the desired duration of the treatment.

In general, recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between 10^4 and 10^{14} pfu/ml, and preferably 10^6 to 10^{10} pfu/ml. The term pfu (plaque-forming unit) represents the infectious power of a particular solution, and it is determined by infection of an appropriate cell culture, and measurement, generally after 5 days, of the number of plaques of infected cells. Methods of determining the pfu titer of a viral solution are well documented in the literature. More particularly, with regard to immunosuppressive agents, the doses and injection methods for these agents vary according to their nature. The adjustment of these parameters is within the competence of those skilled in the art.

The medicinal association according to the invention can be used for the treatment or prevention of numerous pathologies. Depending on the therapeutic gene that is inserted into its adenovirus, it can be used, notably, for the treatment or prevention of genetic diseases ([muscular] dystrophy, cystic fibrosis, etc., neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, ALS, etc.), hyperproliferative diseases (cancers, restenosis, etc.), diseases related to coagulation disorders or to dyslipoproteinemias, diseases related to viral infections (hepatitis, AIDS, etc.), etc.

The present invention also applies to any method of therapeutic treatment that implements the claimed medicinal association.

The present invention will be more fully described with the help of the following examples, which should be regarded as illustrative and not limiting.

Figure 1: Genetic organization of the Ad5 adenovirus. The complete Ad5 sequence is available on the data base and permits those skilled in the art to select or to create any restriction site, and thus to isolate any region of the genome.

Figure 2: Restriction map of the Manhattan strain CAV2 adenovirus (according to the aforementioned Spibey et al.).

Figure 3: Construction of the pAD5-gp19k-ßgal vector.

Figure 4: Construction of the Ad-gp19k-ßgal,ΔE1, ,ΔE3 adenovirus.

General Molecular Biology Techniques

The classic methods used in molecular biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in a cesium chloride gradient, electrophoresis on agarose or acrylamide gels, purification of DNA fragments by electroelution, extraction of proteins with phenol or phenol-chloroform, DNA precipitation in saline medium by ethanol or isopropanol, transformation in *Escherichia coli*, etc. are well known to those skilled in the art and are fully described in the literature [Maniatis T. et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel F.M. et al. (eds), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987].

Type pBR322, pUC plasmids and series M13 phages can be obtained from commercial sources (Bethesda Research Laboratories).

For splicing, the DNA fragments can be separated according to their size by agarose or acrylamide gel electrophoresis, extracted with phenol or by a phenol-chloroform mixture, precipitated with ethanol, then incubated in the presence of T phage DNA ligase (Biolabs) according to the supplier's recommendations.

Filling of the proeminent 5' ends can be effected by the Klenow fragment of DNA Polymerase I of *E. coli* (Biolabs) used according to the manufacturer's recommendations. The destruction of the proeminent 3' ends takes place in the presence of T4 phage DNA polymerase (Biolabs) used according to the manufacturer's recommendations. The destruction of the proeminent 5' ends is carried out by a treatment controlled by the S1 nuclease.

Mutagenesis directed *in vitro* by synthetic oligodeoxynucleotides can be carried out according to the method developed by Taylor et al. [Nucleic Acids Res. 13 (1985) 8749-8764] using the kit distributed by Amersham.

Enzyme amplification of DNA fragments by the so-called PCR method [Polymerase-catalyzed Chain Reaction, Saiki R.K. et al., Science 230 (1985) 1350-1354; Mullis K.B. and Faloona F.A., Meth. Enzym. 155 (1987) 335-350] can be carried out using a "DNA thermal cycler" (Perkin Elmer Cetus) according to the manufacturer's specifications.

Verification of nucleotide sequences can be carried out by the method developed by Sanger et al. [Proc. Natl. Acad. Sci. USA, 74 (1977) 5463-5467) using the kit distributed by Amersham.

Cell Lines Used

In the following examples, the following cells lines have been used or could be used:

- Human embryo kidney line 293 (Graham et al., J. Gen. Virol. 36 (1977) 59). This line contains, notably, integrated into its genome, the left section of the genome of the human adenovirus Ad5 (12%).

- KB Human cell line: Obtained from human epidermal carcinoma, this line is accessible to the ATCC (ref. CCL17) as well as the conditions for its culture.

- HeLa human cell line: Obtained from human epithelium carcinoma, this line is accessible to the ATCC (ref. CCL2) as well as the conditions for its culture.

- MDCK canine cell line: The conditions for culturing these MDCK cells have been described, notably by Macatney et al., Science 44 (1988) 9.

- gm DBP6 cell line (Brough et al., Virology 190 (1992) 624). This line consists of HeLa cells carrying the adenovirus E2 gene under the control of LTR [Long Term Repeat] and MMTV [Mouse Mammary Tumor Virus].

EXAMPLES

Example 1. Construction of defective recombinant adenoviruses comprising a therapeutic gene (the LacZ gene of *E. coli*) under the control of the LTR promotor of the RSV and the gp19k gene under the control of the RSV LTR promoter, both inserted in the E1 region.

These adenoviruses have been constructed by homologous recombination between a plasmid carrying the left section of the Ad5 adenovirus, the two recombinant DNAs and a region of the Ad5 adenovirus (corresponding to protein IX) and the DNA of a defective adenovirus having different deletions.

1. Construction of the pAD5-gp19k-ßgal vector (Figure 3)

1.1 Construction of the pGEM-gp19k plasmid

The pAD5-gp19k-ßgal plasmid contains a cDNA sequence encoding for the adenovirus gp19k protein. This plasmid was constructed as follows. The XbaI fragment of the wild-type adenovirus Ad5

genome containing the E3 region was isolated and cloned at the corresponding site of the pGEM plasmid (Promega) to generate the pGEM-E3 plasmid. The HinfI fragment containing the gp19k encoding sequence (nucleotides 28628 to 29634 of the wild-type Ad5 adenovirus) was then isolated from the pGEM-E3 plasmid. The ends of this fragment were rendered free by the action of the Klenow fragment of DNA polymerase I of *E. coli* (Cf General Molecular Biology Techniques), then the fragment obtained was cloned at the SmaI site of the pGEMzf+ plasmid (Promega).

The plasma obtained was designated pGEM-gp19k (Figure 3).

1.2. Construction of the pAD5-gp19k-ßgal vector

This example describes the construction of a plasmid containing one of the two recombinant DNAs comprising their own promoter, the left section of the adenovirus genome and a supplementary part (protein pIX) permitting homologous recombination. This vector was constructed from the pAd.RSVßGal plasmid as follows.

The pAd.RSVßGal plasmid contains, in the 5'->3' orientation,

- the PvuII fragment corresponding to the left end of the Ad5 adenovirus comprising: the ITR sequence, the replication source, the encapsidation signals and the E1A amplifier;

- the gene encoding for ß-galactosidase under the control of the RSV (Rous sarcoma virus) promoter,

- a second fragment of the Ad5 adenovirus genome, which permits homologous recombination between the pAd.RSVßGal plasmid and adenovirus d1324. The pAD.RSVßGal was described by Stratford-Perricaudet et al. (J. Clin. Invest. 90 (1992) 626).

Plasmid pAd.RSVßGal was first cut by the EagI and ClaI enzymes. This generated a first fragment carrying notably the left part of the Ad5 adenovirus and the LTR promoter of the RSV. In parallel, plasmid pAd.RSVßGal was also cut by the EagI and XabI enzymes. This generated a second type of fragment carrying notably the LTR promoter of the RSV, the LacZ gene, and a fragment of the Ad5 adenovirus, which permits homologous recombination. The ClaI-EagI and EagI-XbaI were then spliced

in the presence of the XbaI-ClaI fragment of the pGEM-gp19k plasmid (example 1.1) carrying the coding sequence of gp19k (Cf Figure 3). The vector obtained, which was called pAD5-gp19k-ßgal, contained, therefore,

- the PvuII fragment corresponding to the left end of the Ad5 adenovirus comprising: the ITR sequence, the replication source, the encapsidation signals and the E1A amplifier;

- the sequence encoding for gp19k under the control of the RSV promoter (of the Rous sarcoma virus);

- the gene encoding for ß-galactosidase under the control of the RSV promoter (of the Rous sarcoma virus), and

- a second fragment of the Ad5 adenovirus genome, which permits homologous recombination.

2. Construction of recombinant adenoviruses

2.1. Construction of a recombinant adenovirus deleted in the E1 region, having two recombinant DNAs inserted in the same orientation, at the level of the E1 region.

The pAD5-gp19k-ßgal vector was linearized and cotransfected with an adenoviral vector that is deficient in the E1 gene, in helper cells (line 293) changing the functions coded by the E1 regions (E1A and E1B) of the adenovirus to the *trans* configuration.

More specifically, the adenovirus Ad-gp19k-ßgal-ΔE1 is obtained by *in vivo* homologous recombination between the Ad-RSVßgal adenovirus (Cf Stratford-Perricaudet et al. cited above) and the pAD5-gp19k-ßgal vector is obtained according to the following protocol: the pAD5-gp19k-ßgal plasmid, linearized by XmnI, and the Ad-RSVßgal adenovirus, linearized by the ClaI enzyme, are cotransfected into line 293 in the presence of calcium phosphate to permit homologous recombination. The recombinant adenoviruses generated are then selected by plate purification. After isolation, the DNA of the recombinant adenovirus is amplified in cell line 293, which leads to a culture supernatant containing the non-purified defective recombinant adenovirus having a concentration of approximately 10^{10} pfu/ml.

Viral particles are generally purified by centrifugation on a cesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). The AD-gp19k-ßgal,ΔE1 adenovirus can be stored at –80°C in 20% glycerol.

2.2. Construction of a recombinant adenovirus deleted in the E1 and E3 regions, having two recombinant DNAs inserted in the same orientation, at the level of the E1 region (Figure 4).

The pAD5-gp19k-ßgal vector was linearized and cotransfected with an adenoviral vector that is deficient in the E1 and E3 genes, in helper cells (line 293) and the functions encoded by the E1 regions (E1A and E1B) of the adenovirus were changed to the *trans* configuration.

More specifically, the adenovirus Ad-gp19k-ßgal-ΔE1,ΔE3 was obtained by *in vivo* homologous recombination between the mutant adenovirus Ad-dl1324 (Thimmappaya et al., Cell 31 (1982) 543) and the pAD5-gp19k-ßgal vector, according to the following protocol: the pAD5-gp19k-ßgal plasmid, linearized by XmnI, and the Ad-RSVßgal adenovirus and the Ad-dl1324 adenovirus, linearized by the ClaI enzyme, were cotransfected into line 293 in the presence of calcium phosphate to permit homologous recombination. The recombinant adenoviruses generated were then selected by plate purification. After isolation, the DNA of the recombinant adenovirus was amplified in cell line 293, which produced a culture supernatant containing the non-purified defective recombinant adenovirus having a concentration of approximately 10^{10} pfu/ml.

Viral particles are generally purified by centrifugation on a cesium chloride gradient according to known techniques (see, in particular, Graham et al., Virology 52 (1973) 456). The genome of the recombinant adenovirus is then verified by Southern blot analysis. The AD-gp19k-ßgal,ΔE1, ΔE3 adenovirus can be stored at –80°C in 20% glycerol.

Example 2: Detection of the immunoprotective activity of the medicinal association according to the invention.

Sixty DBA/2 adult female mice were randomly divided into 6 groups of mice treated according to the following respective injection protocols:

- GROUP 1a:

This group received an intraocular injection of 10 μg of anti-CD3 monoclonal antibody on days –2, -1, 1, 2, 3, 4 and 5 with an intravenous injection of 4.10^9 pfu of the Ad-RSVßgal virus on day zero (Cf Stratford-Perricaudet et al. cited above).

- GROUP 1b:

This group received the same treatment as group 1a using as the virus 4.10^9 pfu of the Ad-gp19k-ßgal virus (Figure 4).

- GROUP 2a:

This group received an intrapetitional [sic – possible misprint for "intraperitoneal"] injection of 250 μg of anti-CD4 monoclonal antibody on days –2, -1, 1, 4, 7 with an intravenous injection of 4.10^9 pfu of the Ad-RSV ß gal virus on day zero.

- GROUP 2b:

This group received the same treatment as group 2a using as the virus 4.10^9 pfu of the Ad gp 19k-ßgal virus.

- GROUP 3a:

This group received an intravenous injection of 4.10^9 pfu of Ad-ßgal without the combined administration of an immunosuppressive agent.

- GROUP 3b:

This group received an intravenous injection of 4.10^9 pfu of Ad-gp19k-ßgal without the combined administration of an immunosuppressive agent.

At different times, two animals from each group were sacrificed and their livers and spleens were extracted.

2.1- Determination by FACS [fluorescence activated cell sorter] analysis of the % of splenocytes having the antigen 14 days post-injection.

The spleens were crushed and the splenocytes were extracted. A sample was subjected to FACS analysis to determine the distribution of immune cells. Table I below summarizes the results.

[Page prior to modification]

	Group 3 a Ad-ßgal		Group 3 b Ad-ßgal-gp19K		Group 3 a CD3/Ad-ßgal		Group 2 a CD4/Ad-ßgal	
	% of cells expressing ßgal on the surface of the cell							
CD3	20.4	17.5	20.6	21	5.4	6.1	12	10.3
CD4	13.4	12.6	15.3	16.8	4.4	5.1	2.7	4.1
CD8	5.5	5.5	6.1	6	20.2	23	7.9	6.7

TABLE I

Note the inhibiting effect of the anti-CD3 and anti-CD4 immunosuppressive agents on antibody production.

2.2- Cytotoxicity of splenocytes stimulated 14 days post-injection

The remainder of the cells extracted from the liver were stimulated in the presence of P815-ßgal cells, infected with Ad-ßgal at a MOI [Multiplicity of Infection] of 100 pfu, to amplify any CTL [cytotoxic T-lymphocyte] clone recognizing the ß-galactosidase antigens. After 4 days of stimulation, the cytotoxic activity of the splenocytes was determined, using a ^{51}Cr release cytotoxic test, with labeled P815-ß gal cells used as the target cells. The results are presented in Table II below.

Group 2a (anti-CD4/Ad-ßgal)	Low
Group 2b (anti-CD4/Ad gp19k-ßgal)	Undetectable
Group 3a (Ad-ßgal)	High
Group 3b (Ad-gp19k-ßgal)	Average

TABLE II

Only the group treated according to the invention, namely group 2b, was not associated with any cytotoxic activity.

2.3 Expression of ß-galactosidase activity in the liver after 14 days

The livers were sectioned and stained with X-gal to reveal ß-galactosidase activity and eosin to reveal the section's histology. The results are presented in Table III below.

	Number of cells expressing ßgal	
	14 days	31 days
Group 2a: (anti-CD4/Ad-ßgal)	1	1
Group 2b: (anti-CD4/Ad gp19k-ßgal)	250	50
Group 3a: (Ad-ßgal)	3	0
Group 3b: (Ad-gp19k-ßgal)	25	0

TABLE III

From the results shown above, it is obvious that the injection of anti-CD4 antibodies combined with an injection of Adgp-19k-ßgal induced a clearly prolonged expression of the gene in question. Thirty days after the injections, significant ß-galactosidase activity can be observed in the case of group 2b. This prolongation, which can be interpreted as the result of a tolerance phenomenon induced according to the invention, is clearly superior to that which might be expected from the simple juxtaposition of the respective effects of the anti-CD4 immunosuppressant and the Ad gp198-ßgal recombinant adenovirus.

In addition, no inflammatory reaction was observed during this 30-day period in the case of Group 2b.

CLAIMS

1. Medicinal combination of at least an immunosuppressive agent and at least a recombinant adenovirus the genome of which includes a first recombinant DNA containing a therapeutic gene and a second recombinant DNA containing an immunoprotective gene, for consecutive, intermittent and/or simultaneous use over time, useful for exogenic *in vivo* and/or *ex-vivo* transfections.

2. Medicinal combination according to Claim 1 characterized in that the immunosuppressive agent is preferably selected from cyclosporin, FK506, azathioprine, corticosteroids and monoclonal or polyclonal antibodies.

3. Medicinal combination according to Claim 2 characterized in that it involves antibodies that are capable of inactivating immune molecules and causing the destruction of the immune cells having these molecules.

4. Medicinal combination according to Claim 3 characterized in that the antibody is selected from anti-CD4, -CD2, -CD3, -CD8, -CD28, -B7, -ICAM-1, -LFA-1 and CTLA4Ig.

5. Medicinal combination according to one of the preceding claims characterized in that the therapeutic gene encodes for a therapeutic protein.

6. Medicinal combination according to one of Claims 1 to 4 characterized in that the therapeutic gene encodes for a therapeutic RNA.

7. Medicinal combination according to one of the preceding claims characterized in that the immunoprotective gene is a gene whose product acts on the activity of the major histocompatibility complex (MHC) or on the activity of cytokines.

8. Medicinal combination according to Claim 7 characterized in that the immunoprotective gene is a gene whose product at least partially inhibits the expression of MHC proteins or the introduction of antigens.

9. Medicinal combination according to one of the preceding claims characterized in that the immunoprotective gene is selected from the gp19k gene of the adenovirus, the ICP47 gene of the herpes virus, or the UL18 gene of the cytomegalovirus.

10. Medicinal combination according to one of the preceding claims characterized in that the two recombinant DNAs of the adenovirus genome constitute a unique transcriptional entity.

11. Medicinal combination according to one of the preceding claims characterized in that each one of the two recombinant DNAs has an identical or different transcriptional promoter.

12. Medicinal combination according to Claim 11 characterized in that the two recombinant DNAs are inserted in the same orientation.

13. Medicinal combination according to Claim 11 characterized in that the two recombinant DNAs are inserted in opposite orientations.

14. Medicinal combination according to one of the preceding claims characterized in that the two recombinant DNAs are inserted in the same site of the adenovirus genome, preferably at the level of regions E1, E3 or E4.

15. Medicinal combination according to Claim 14 characterized in that the two recombinant DNAs are inserted at the level of region E1.

16. Medicinal combination according to one of Claims 1 to 13 characterized in that the two recombinant DNAs are inserted into different sites of the adenovirus genome.

17. Medicinal combination according to Claim 16 characterized in that one of the recombinant DNAs is inserted at the level of the E1 region and the other at the level of the E3 or E4 region.

18. Medicinal combination according to one of the preceding claims characterized in that the adenovirus is a defective recombinant adenovirus comprising ITR sequences, a sequence that permits encapsidation, and has a deletion of all or part of the E1 and E4 genes.

19. Medicinal combination according to Claim 18 characterized in that it is an adenovirus comprising ITR sequences, a sequence that permits encapsidation and has a deletion of all or part of the E1, E3 and E4 genes.

20. Medicinal combination according to one of Claims 1 to 19 characterized in that it involves an adenovirus of which the genome is deleted of all or part of genes E1, E3, L5 and E4.

21. Medicinal combination according to one of the preceding claims characterized in that the recombinant adenovirus is of human, animal or mixed origin.

22. Medicinal combination according to Claim 21 characterized in that the recombinant adenoviruses of human origin are selected from those classified in Group C, preferably from type 2 or type 5 recombinant adenoviruses (Ad 2 or Ad 5).

23. Medicinal combination according to Claim 22 characterized in that the adenoviruses of animals origin are selected from adenoviruses of canine, bovine, murine, ovine, porcine, avian and simian origin.

24. Medicinal combination according to one of the preceding claims characterized in that the immunosuppressive agent is injected before and after the injection of the adenovirus.

25. Medicinal combination according to one of the preceding claims characterized in that the immunosuppressive agent and the recombinant adenovirus are injected simultaneously.

[Modified page]

	Group 3 a Ad-ßgal		Group 3 b Ad-ßgal-gp19K		Group 1 a anti CD3/ Ad-ßgal		Group 2 a anti CD4/ Ad-ßgal	
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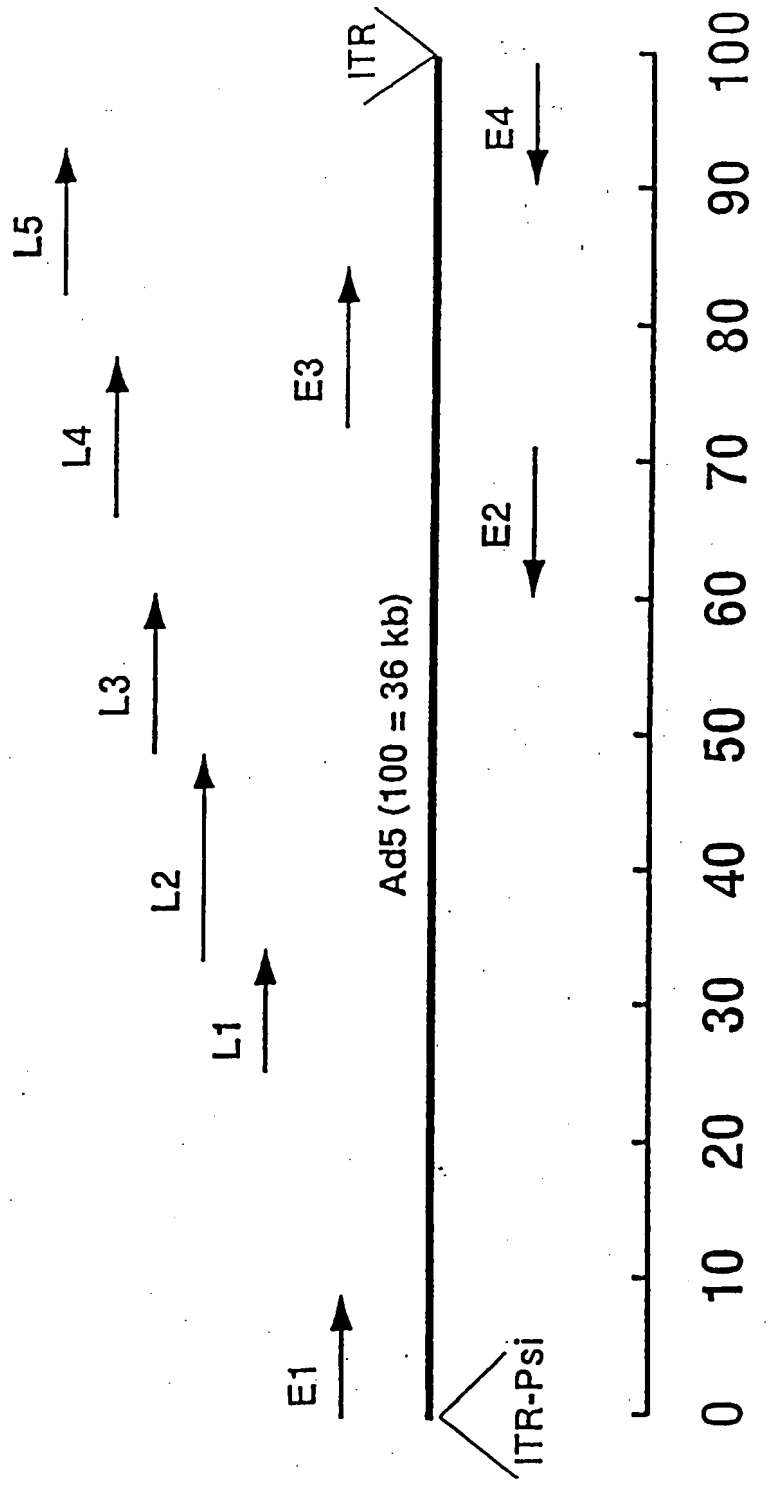
Group 3b (Ad-gp19k-ßgal)	Average

TABLE II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E4
L5
E3
L4
E2
L3
L2
Ad5 (100 = 36 kb)
L1
E1
ITR-Psi
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10
20
30
40
50
60
70
80
90
100

Figure 1

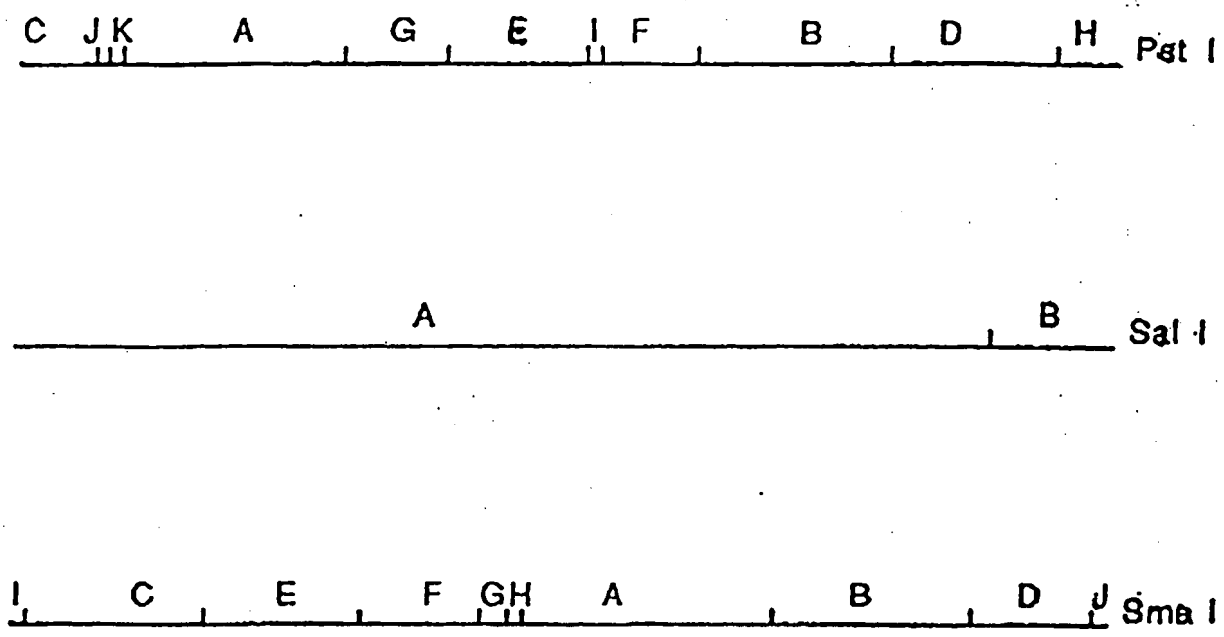
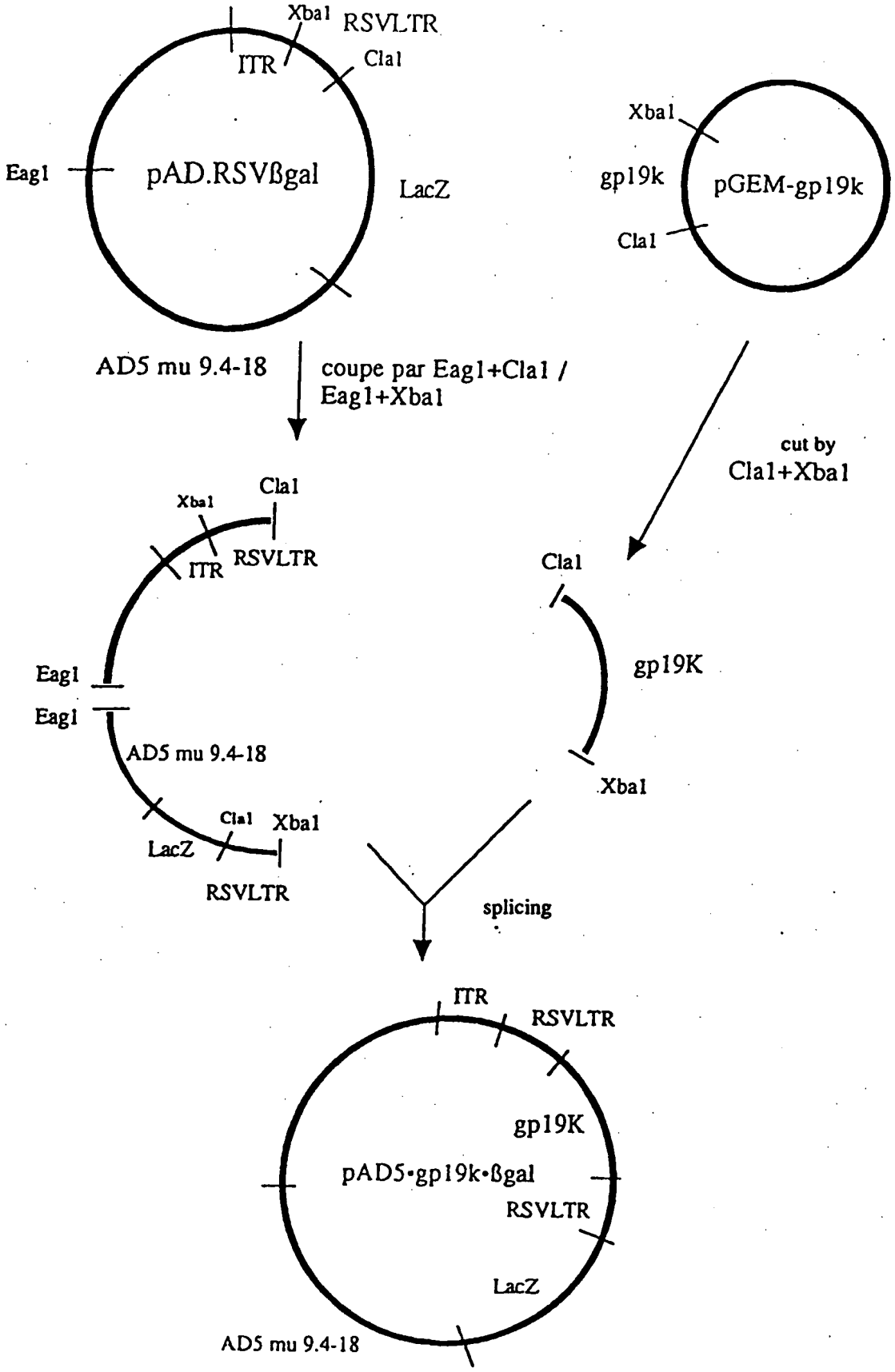


Figure 2


Xba1
RSVLTR
ITR
Cla1
Eag1
pAD.RSVßgal
LacZ
Xba1
gp19k
pGEM-gp19k
Cla1
AD5 mu 9.4-18
coupe par Eag1+Cla1 /
Eag1+Xba1
cut by
Cla1+Xba1
Cla1
Xba1
ITR
RSVLTR
Cla1
gp19K
Eag1
Eag1
Xba1
AD5 mu 9.4-18
Cla1
Xba1
LacZ
RSVLTR
splicing
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gp19K
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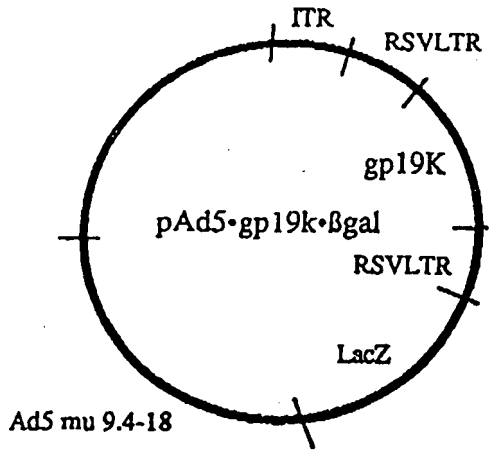
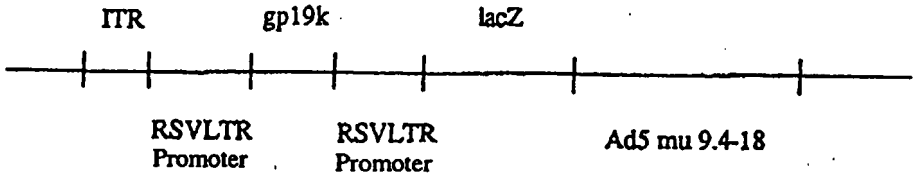
RSVLTR
LacZ
AD5 mu 9.4-18



Linearization



Recombination

D1324 genome

Ad-gp19K-lacZ